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Diversity of TEM-52 extended-spectrum β -lactamase-producing non-typhoidal *Salmonella* isolates in Korea

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Objectives: Extended-spectrum β -lactamase (ESBL)-producing non-typhoidal *Salmonella* (NTS) isolates in Korea were characterized.

Patients and methods: Five isolates of ESBL-producing NTS were isolated from stool specimens of three infants and two adults with diarrhoea. Two infants acquired the infection in the community, and three other infections were hospital acquired.

Results: The isolates were one each of serovars Saintpaul, Stanley and Agona, and two Enteritidis. Cell sonicates of the isolates hydrolysed cefotaxime more efficiently than ceftazidime, and had β -lactamase bands of approximate isoelectric points 6.0 and 7.4. Sequencing revealed that the β -lactamases were TEM-52 and an OXA type. The *bla*_{OXA} gene was located on a class 1 integron. Cefotaxime resistance, associated with TEM-52, was transferred by conjugation. Identical pulsed-field gel electrophoresis patterns of *Xba*I-digested genomic DNA were observed in initially β -lactam-susceptible serovar Agona isolates and subsequent ESBL-producing isolates from an infant, and in two isolates of serovar Enteritidis from two different patients.

Conclusions: This study suggests that TEM-52-producing NTS is spreading both clonally and horizontally in Korea.

Keywords: enteritis, class 1 integron, OXA-type β -lactamase

Introduction

Non-typhoidal *Salmonella* (NTS) is one of the most important enteric pathogens worldwide. Antimicrobial treatment is required for NTS gastroenteritis only when the patients are of extreme ages, or when they have underlying diseases.¹ Extended-spectrum β -lactamase (ESBL) production is a particular concern, as expanded-spectrum cephalosporins are the drugs of choice for children because they cannot be treated with fluoroquinolones. Moreover, the ESBL genes reside on conjugative plasmids and have the potential to spread to other bacteria. TEM- and SHV-type ESBLs were first reported in Korea in 1997,² and the prevalent type was TEM-52.³ The aim of this study was to determine the phenotypic and genetic characteristics of ESBL-producing NTS strains isolated in Korea.

Materials and methods

Strains

NTS strains were isolated from stool specimens during 1995–1997 from five in-patients with diarrhoea at a tertiary-care hospital. The species

were identified by conventional methods and the serovar was determined by the National Institute of Health, Korea.

Antimicrobial susceptibility testing and β -lactamase investigation

The disc diffusion test⁴ was performed using commercial discs and Mueller–Hinton agar (Becton Dickinson, Cockeysville, MD, USA). ESBL production was determined by the double disc synergy test and confirmed using the NCCLS broth dilution method.⁴

MICs of β -lactams were determined using an agar dilution test⁴ with ampicillin and cefalothin (Sigma Chemical, St Louis, MO, USA), piperacillin and tazobactam (Wyeth, Pearl River, NY, USA), cefotaxime (Aventis, Frankfurt, Germany), ceftazidime and clavulanic acid (Glaxo-SmithKline, Greenford, UK), aztreonam (Bristol-Myers Squibb, Princeton, NJ, USA), and cefoxitin and imipenem (Merck, Sharp & Dohme, Rahway, NJ, USA).

Isoelectric points (pI) of β -lactamases were determined by loading cell sonicates on pre-cast gels and separating them by use of a Thermo-Flow Electrophoresis Temperature Control System (Novel Experimental Technology, San Diego, CA, USA). The bands were visualized using

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Table 1. Clinical features of the patients with ESBL-producing NTS isolation from stool

Case no.	Sex/age (years)	Underlying diseases	Present illness	<i>Salmonella</i> serovar	Persistent isolation	Treatment
1	M/0.4	none	diarrhoea	Saintpaul	7 weeks	cefaclor, co-trimoxazole
2	F/1	agenesis of corpus callosum	diarrhoea, pneumonic consolidation	Stanley	1 week	cefoperazone, amikacin
3	M/1	none	diarrhoea	Agona (S), Agona (R)	18 days, 13 days	cefaclor, co-trimoxazole
4	F/25	osteosarcoma	diarrhoea 1 month after admission	Enteritidis	ND	cefotaxime
5	F/51	mitral valve replacement	diarrhoea 1 month after admission	Enteritidis	ND	cefoperazone, tobramycin

M, male; F, female; ND, not determined; S, β -lactam susceptible; R, β -lactam resistant.

nitrocefin solution. β -Lactamase activities of the cell sonicates were determined using a model UV-2401 spectrophotometer (Shimadzu, Tokyo, Japan) with 100 μ M solutions of β -lactams in 50 mM phosphate buffer (pH 7.0) at 30°C. Results were expressed as relative activity versus that observed with penicillin G.⁵

PCR amplification and sequencing

Alleles of *bla*_{TEM} and *bla*_{SHV} were detected using previously reported primers and reaction conditions.² For *bla*_{TEM} sequencing, a PCR product of 1080 bp was amplified using primers: TEM-SF, 5'-ATAAAAT-TCTTGAAGACGAAA-3' and TEM-SR, 5'-GACAGTTACCAAT-GCTTAATC-3'. A PCR product from the class 1 integron was obtained using previously reported primers and reaction conditions.⁶ The nucleotide sequence was determined by direct sequencing on an ABI 3700 Automatic sequencer (Perkin-Elmer, Foster City, CA, USA). Both strands were sequenced twice with independent amplicons.

Plasmid study and pulsed-field gel electrophoresis (PFGE)

Plasmids were isolated by the alkaline lysis method, and after electrophoresis their sizes were estimated by comparison with those of *Escherichia coli* strain V517. Conjugation was performed by the broth mating method using rifampicin-resistant *E. coli* strain RG 488 and nalidixic acid-resistant *E. coli* strain RG 176 as recipients.

Genomic DNA of the NTS isolates was digested using *Xba*I, and the bands were separated using a CHEF-DRII system according to the manufacturer's instruction (Bio-Rad, Hercules, CA, USA). The band patterns were compared visually.

Results and discussion

Clinical findings

Two infants with no underlying diseases were admitted because of diarrhoea, but one infant and two adults developed diarrhoea during hospitalization to treat underlying diseases. One strain each of *Salmonella* serovars Saintpaul, Stanley and Agona were isolated from three patients, and two strains of serovar Enteritidis were isolated from two different patients (Table 1). Sporadic or nosocomial outbreaks of ESBL-producing NTS infections have been reported in

many countries. It was reported that four of 26 consecutive isolates of community-acquired serovar Typhimurium in Turkey produced ESBLs.⁷

Except for the initial isolates of serovar Agona, all other isolates produced ESBLs. Strains of serovar Agona, which were susceptible to β -lactams, aminoglycosides, co-trimoxazole and ofloxacin, were isolated initially from one infant, and persisted until the eighteenth hospital day culture. The patient was treated with cefaclor and ESBL-producing strains of the same serovar were isolated from a specimen taken 41 days after the initial isolation of the susceptible strains.

Follow-up stool culture from three infants showed persistence of the same serovar for 1–7 weeks. The carriage was probably prolonged because all of the patients were treated with antimicrobial agents for other diseases, none of which was recommended for the treatment of NTS enteritis.

Antimicrobial resistance and β -lactamase investigation

In our study, all ESBL-producing isolates and the transconjugants were resistant to multiple β -lactams, but susceptible to ceftazidime, imipenem and ofloxacin. Susceptibility to aminoglycosides and co-trimoxazole was variable: isolates and the transconjugants of serovars Saintpaul and Enteritidis were resistant to amikacin, netilmicin and tobramycin, but susceptible to gentamicin; serovars Stanley and Agona were resistant to gentamicin and tobramycin, but susceptible to amikacin and netilmicin. All isolates and the transconjugants except serovar Stanley were resistant to co-trimoxazole.

The MICs of cefotaxime were equal to or lower than those of ceftazidime for all the ESBL-producing isolates tested, but cefotaxime was hydrolysed more efficiently than ceftazidime, as reported in other TEM-52-producing isolates.⁸ The MICs of all β -lactams were decreased by the addition of clavulanic acid, but those of piperacillin did not decrease significantly after addition of tazobactam in three isolates.

Isoelectric focusing showed β -lactamase bands of pI ~5.4 and ~6.0 in two isolates, suggesting TEM-type enzymes, but in three isolates with high MICs of piperacillin-tazobactam, bands of pI ~6.0 and ~7.4 were observed indicating the presence of other β -lactamases (Table 2).

Table 2. Characteristics of ESBL-producing NTS isolates and their transconjugants

Case no., strain no.		MIC (mg/L) ^a									Relative hydrolysis (%) ^b				PCR	
		CTX	CTX + CLV	CAZ	CAZ + CLV	ATM	ATM + CLV	PIP	PIP + CLV	PIP + TAZ	CTX	CAZ	ATM	pI	bla _{TEM}	bla _{OXA}
(1) <i>Salmonella</i> Saintpaul, 95/4/4199	CI	128	1	128	2	16	0.5	>256	16	>128	66	2	2	6.0, 7.4	+	+
	TC	32	0.25	32	0.5	8	0.25	>256	8	128	97	6	6	6.0, 7.4	+	+
(2) <i>Salmonella</i> Stanley, 96/7/4034	CI	256	0.5	256	2	32	0.5	>256	8	4	56	2	4	5.4, 6.0	+	–
	TC	16	0.12	32	0.5	8	0.12	>256	2	2	76	4	<1	5.4, 6.0	+	–
(3) <i>Salmonella</i> Agona, 96/9/4280 (S)	CI	0.12	0.12	0.5	0.5	0.12	0.12	2	2	4	NT	NT	NT	NT	NT	NT
<i>Salmonella</i> Agona, 96/10/4368 (R)	CI	128	0.5	>256	2	16	0.25	>256	4	4	61	4	<1	5.4, 6.0	+	–
	TC	32	0.12	32	0.5	4	0.12	>256	4	1	54	4	2	5.4, 6.0	+	–
(4) <i>Salmonella</i> Enteritidis, 97/4/4434	CI	128	1	128	2	16	0.5	>256	16	>128	33	5	3	6.0, 7.4	+	+
	TC	32	0.25	32	0.5	4	0.12	>256	8	16	90	8	3	6.0, 7.4	+	+
(5) <i>Salmonella</i> Enteritidis, 97/8/4419	CI	32	1	128	1	8	0.12	>256	4	>128	85	6	9	6.0, 7.4	+	+
	TC	32	0.25	16	0.5	4	0.12	>256	4	16	123	15	10	6.0, 7.4	+	+

CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; PIP, piperacillin; CLV, clavulanic acid; TAZ, tazobactam; CI, clinical isolate; TC, transconjugants; S, susceptible isolate; R, resistant isolate; NT, not tested.

^aMICs (mg/L) of ampicillin, cefalothin, cefoxitin and imipenem were >256, >256, ≤4 and ≤0.25, respectively, against all ESBL-producing isolates and their transconjugants. All ESBL-producing isolates and the transconjugants were susceptible to ofloxacin.

^bRelative hydrolysis compared with that of penicillin G.

Genetic characteristics

By PCR analysis, alleles of *bla*_{TEM} were detected in all of the strains, and those of *bla*_{OXA} in three strains with a β -lactamase band of pI ~7.4 (Table 2). Sequencing of the *bla*_{TEM} amplicon showed deduced amino acid changes of Glu-104→Lys, Met-182→Thr and Gly-238→Ser from TEM-1, which correspond to TEM-52 β -lactamase (GenBank accession no. AF126444).

Presence of a class 1 integron was detected by PCR in strains with β -lactamase band of pI~7.4. Sequencing showed that the integron carried *aacA4*, an unknown *orf*, and *bla*_{OXA1} (GenBank accession no. AY220520). *bla*_{OXA1} (not *bla*_{OXA-1}) was identical to that reported from a sludge sample (GenBank accession no. AY139600). Class 1 integrons, carrying various resistance gene cassettes, were reported in 76% of serovar Typhimurium DT104 and 48% of the non-phage-typeable strains in Spain.⁹

Our isolates had plasmids of >120 MDa (data not shown). The plasmids carrying ESBL genes are usually large in size, i.e. \geq 80 kb, although one study showed that *bla*_{TEM-52} gene was carried on a mobilizable plasmid of 13.5 kb in a *Klebsiella pneumoniae* isolate.⁸ The resistance to expanded-spectrum cephalosporins was co-transferred to recipients together with resistance to aminoglycosides and cotrimoxazole, if present. These results indicate that *bla*_{TEM-52}-carrying plasmids with diverse genetic characteristics had been spreading among the NTS.

Epidemiological features

ESBL-producing salmonellae are extremely rare, but recently their prevalence was reported to be 3.4% in western Pacific countries.¹⁰ TEM-52 ESBL in NTS was first found in a Yugoslavian patient. Detection of TEM-52-producing salmonellae is not unusual in Korea, as TEM-52-producing *E. coli* and *K. pneumoniae* are prevalent.³ However, it is interesting that the five isolates belonged to four different serovars, although the most frequently isolated serovars were Typhimurium and Enteritidis (data not shown).

Two serovar Enteritidis strains isolated from two different patients 4 months apart had an identical antimicrobial resistance pattern and an identical PFGE pattern, suggesting that they belonged to an identical clone. The ESBL-producing and -non-producing strains of serovar Agona from a patient had an identical PFGE pattern, suggesting acquisition of resistance.

In conclusion, isolation of five strains of TEM-52 ESBL-producing NTS with four different serovars, three different resistance patterns and different genetic characteristics suggests that resistance is

spreading both clonally and horizontally to NTS in Korea, not only in hospital, but also in the community.

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